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The Immunomodulation Effect Of Purified Resveratrol Extracted From Black Grape Skin Cultivated In Iraq

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Abstract

The Iraqi black grape is rich with active ingredient that regarded a promise phytochemical indicated to treat and protect many disease and human disordered as well as acted as immune boosting ; this natural substance called (Resveratrol). The study employed extraction, purification, analysis for insuring its purity, and evaluate the purified resveratrol activity on normal human lymphocytes culture as an immune boosting by affecting the interleukins level causing immune modulation effects**.**

Results showed that the black grape skin contain 7mg%W/W resveratrol extracted as two isomers(cis & trans)as pure crystals. The resulting crystals purity was insured in comparison with standard resveratrol Sigma^{R}by (HPLC), (CHO) checking analysis , and the functional groups by infrared FTIR test, all tests as standard resveratrol assay values. The immune modulation of this purified natural substance was obvious through decreasinglevel of IL 2 & IL 12 significantly within 2&4 hours, while the expression of IL10 started after 4 hours exposure with and dcreasing in IL-6 level at low concentrations of resveratrol significantly

Conclusion: black grape skin cultivated in Iraq is rich with resveratrol that has the ability to regulate and strengthen with boosting the immune system, to stand against diseases and fight abnormal cells in the body, as it works as an immunomodulation agent.

Key words: Iraqi black grape, resveratrol,immune modulation, IL-12, IL-10.

Introduction

Resveratrol (3,4',5-trihydroxystilbene) is a stilbene compound produced in higher plants as a phytoalexin in response to stress and fungal infections found in various fruits and vegetables and is abundant in black grape skin (Vitisvinifera) and has broad effects acted to inhibit different cancer cells through cell cycle modulation beside ,it is a potent antioxidant[1] , anti-

inflammatory, anti-platelets aggregation and cardio-protective agent[2]. The beneficial effects of this dietary ingredient had been attributed to the Polyphenolic structure that act as potent antioxidant and free radical scavenging agent.In addition to various biochemical, biological and pharmacological activities, resveratrol has been found to exhibit numerous immune-modulatory activities such as suppression of lymphocyte proliferation, changes in cell-mediated cytotoxicity, cytokine production or induction of apoptosis[3]. Also, resveratrol was reported to inhibit production of nitric oxide (NO) and tumor necrosis factor (TNF-a) from macrophages. Many researches indicated that resveratrol possessed anti-inflammatory function, and almost all reports stated its immune-suppressive effect[4].

The Iraqi black grape isrich with resveratrol regarded as the most promise phytochemicals[5]. The purified resveratrol occurs in two isomers form (Cis and trans-3,4',5-trihydroxystilbene) and Few studies have been done about resveratrol's human benefits and its effect on lymphocyte culture function. The object of this study as a first work done in Iraq, was to elucidate the in vitro immune effects of different concentrations of resveratrol (purified in Iraq in comparison with commercial standard) on normal human peripheral blood lymphocytes proliferation and cytokine release for investigate Resveratrol Immunomodulation effects.

Material and Method

1-Resveratrol purification from black grape skin[6]:

Resveratrol purification from black grape skin was published in[6], briefly; the fresh skin grapes(1/2kg) had been kept for three days in a state of ethanolic 80% cold dark maceration. With aid of 10%HCl, an acid hydrolysis was done at 60°C for half an hour, then filtered and the filtrate was extracted with chloroform to be finally evaporated till dryness. The residue was proceeding on silca gel G60 column with mobile phase is benzene: methanol : acetic acid , 20:4:1. Another process for purification was followed by preparative Thin Layer Chromatography (P.T.L.C.). An amorphous off white crystals were formed, collected rapidly in cool, dark place and kept in umber reservoir at –20°C. The crystals purity was examined by: U.V. absorption,TLC, HPLC method, FTIR assay, CHN assay content. These tests included the following

A- High-efficiency liquid chromatographic examination of the purified substance and the standard substance was carried out at the Ministry of Science and Technology under the following conditions

Column: C18 – reverse phase

Mobile phase: acetonitrile: water, 60: 40.

Flow rate : 0.6 ml/min.
Standard concentration : 3 mg / 5 ml.
Sample concentration : 3 mg / 5 ml (both standard and sample were dissolved in absolute ethanol.
Wave length: 307 nm. The detector is a spectrum-photo-detector.

2-**ImmunomodulationDetermination(in vitro)**

To determinein vitro immune effects for resveratrol extracted from vitisvinifera; lymphocytes culturing and viable counting was employed in each step; lymphocytes proliferation(MTT Assay) was estimated.Finally Level of different cytokines were estimated to investigate the effectof the purified resveratrol as immune-modulating agent

i- Sample collection

Peripheral venous blood were taken from 8healthy volunteers with age range (25-35) years had never taken medication at least 10 days.The Lymphocyte separation fluid(lymphprep; specific gravity=1.077g/l, was used to separate the normal lymphocytes in general protochol[7] for separation. The isolated lymphocyte cells were suspended in RPMI-1640 medium supplemented with 10% fetal calf serum, containing100 units/ml penicillin, and 100µg/ml streptomycin, then transferred into appropriate tissue culture flask and incubated for 18 hours at 37^0C in 5% CO_2 incubator.

ii- Lymphocytes proliferation Measurement by MTT Assay [8]:-

Resveratrol in serial dilution manner had been prepared to get 12 concentrations (ranging from 2.5 down to 0.01) mg/ml, and sterilized with 0.22 µm Millipore filter.Aliquot of 100µl suspended cells was seeded in each of the 96 well micro-titerplate, (10^4cell/well), then 100 µl from each purified extract concentration was added in triplicate manner. The untreated lymphocyte cells suspended in medium represented the negative control. At the end of exposure time incubation in a CO_2 incubatorat $37C^0$, all wells were subjected to 50 µl of MTT dye(2mg/ml) then incubate for further 4 hours. The MTT-formazan crystals had been formed by live cellsonly. Formazan crystals were dissolved with100µl DMSO added to all wells and the absorbance at 620 nm was recorded by ELISA reader.Viable cell Lymphocytes as a percentage was calculated as followed:

[Absorbance of the test /Absorbance of negative control]X 100.

Acomparison between the results of the extracted resveratrol at different concentrations were statistically calculated to choose the most effective dosages of each extract that may cause lymphocytes proliferation that to be used in further experiment as immune-modulator.

iii- Evaluation of Interleukins in culture supernatants:

Determination the effect of the purified Resveratrol on cytokines IL2, IL6, IL10 and IL12 Level, This assessment of thesecytokines (interleukins) levels extracted by normal human lymphocytes after exposure to three selected resveratrol concentrations(100,30 , and 6)µg/ml was employed for two time intervalsp2 and 4 hours**.. Lymphocytes were Isolation and Culturingaccording to reference**protocol[9]. The general method for lymphocyte isolation and culturing was employed as mentioned in previous section; each isolated sample lymphocytes were re-suspended in 3 ml RPMI 1640 medium.

Work Procedure

-Measuring 500µl isolated lymphocyte suspended cells were seeded in each of the 24 well tissue culture plate($1X10^4$cell/well). Each sample in all treatments were applied in triplicate, to be incubated at least for 2 hours in a CO_2incubator before treatments in order to cells rest after isolation.Three selected resveratrol concentrations were chosen, these are: (100,30 , and 6)µg/ml as they had different range in proliferative effect for the lymphocytes in the previous step mentioned above. All solutions were sterilized with 0.22 µm Millipore filter.

-From each resveratrol concentration aliquot of 500 milliliter was added to each well in duplicate. Negative control represented by untreated cells suspended in growth medium was included, then the plates were incubated for 2 hours and 4 hours, in CO_2 incubator at $37C^0$.

-At the end of interval times all wells were aspirated and transferred in separated vacuum tubes labeled for each sample and centrifuged for 20 minutes at 2000 rpm. The pellets were resuspended in RPMI medium and stored at-20 ^{0}C. The supernatants of each tube were estimated for. Levels of IL2, IL6, IL10 and IL12 determined by commercial ELISA kits from Abcam according to the protocol of the manufacturer after two periods of incubation time (2 hours and 4 hours).Concentrations of these interleukins in each sample were measured according to straight line equation of the plotted standard curve for each cytokine concentrations against their absorbance at 450nm.

Statistical Analysis[10]:

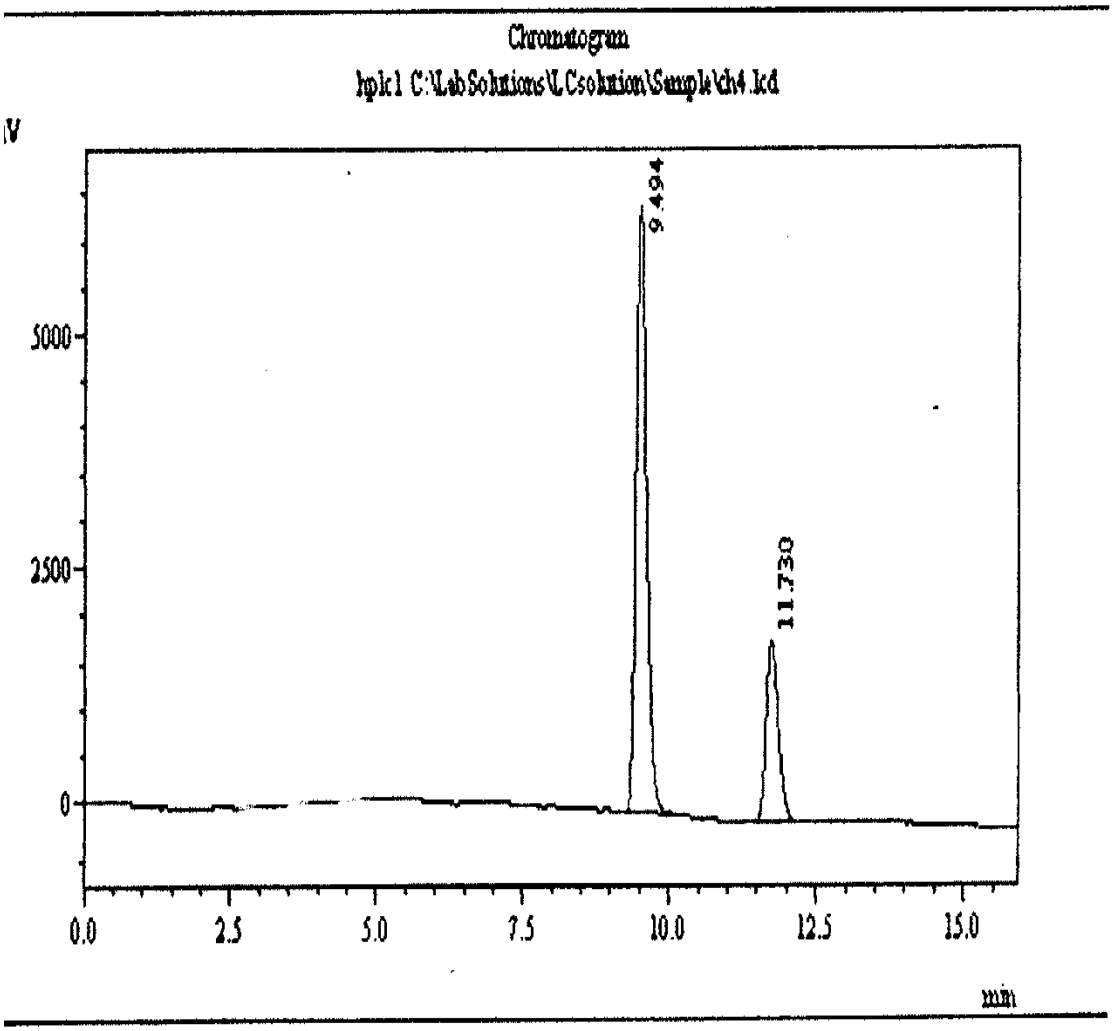
The Statistical Analysis System- SAS (2012) program was used to detect the effect of difference factors in study parameters. Least significant difference –LSD test (Analysis of Variation-ANOVA) was used to significant compare between means in this study.

Result

1-Resveratrol Extraction and Purification

The pure resveratrol extracted from locally grown black grape had been registered at Central organization for standardization and quality control – industrial property division/ Ministry of Planning/Republic of Iraqas a Iq patent with number of 4579at 24/5/2016.

Iraqi black grape skinyields(70mg/kg) Resveratrolas pure crystals. The resulting crystals purity was insured by different tests and comparison with standard resveratrol Sigma^{R}. These purity checking test(HPLC)as in figure -1 and figure- 2, (CHO) analysis as in figure -3, and check the functional groups by infrared FTIR as in figure-4 ,figure-5. Black grapes grown in Iraq is considered as a rich content of this natural stilbene than that had been mentioned in the foreign sources

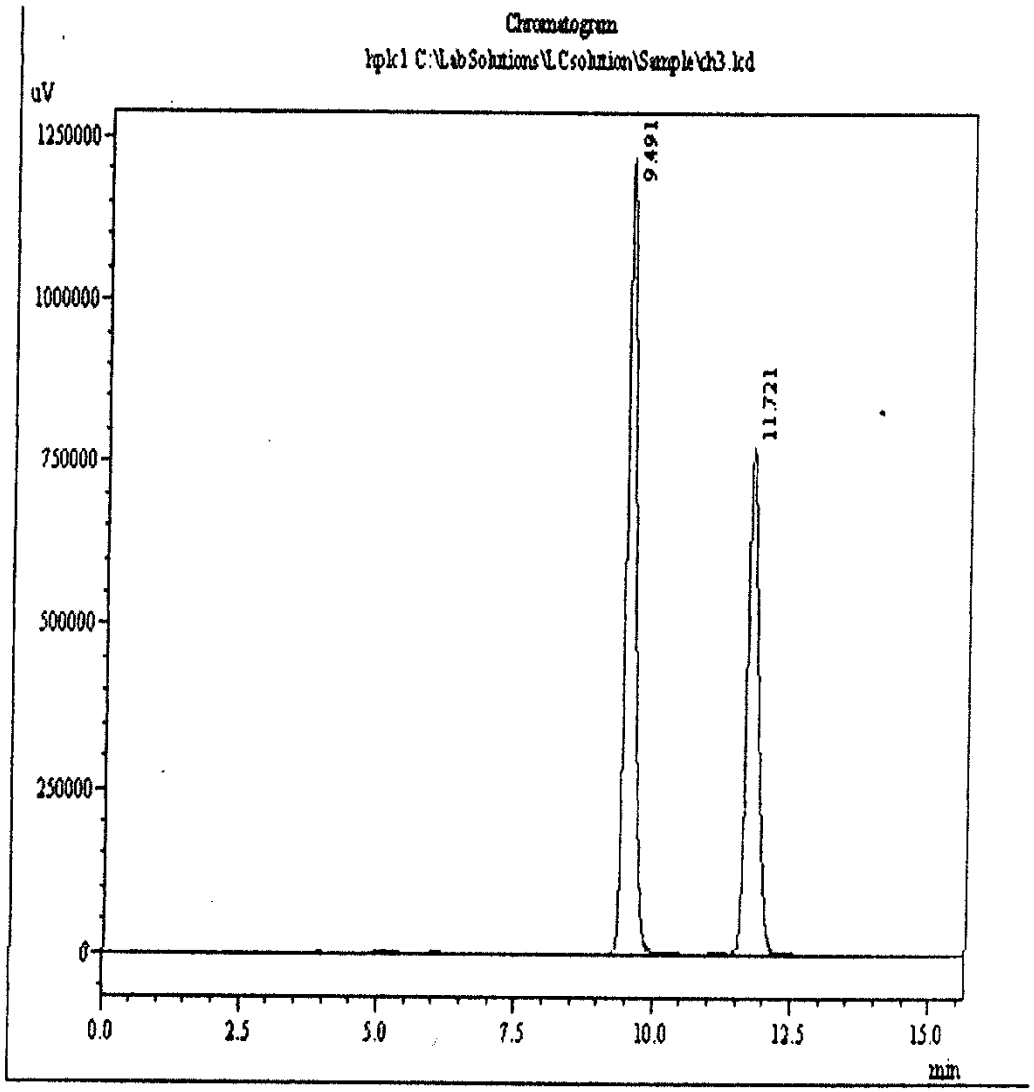


Quantitative Results

A

Name	Ret. Time	Area	Height	Conc.	Area %
RT9.494 Trans	9.494	81791	6476	0.000	53.604
RT11.730 cis	11.730	28343	1934	0.000	18.576

Figure1- HPLC Chromatogram for the Extracted Resveratrol(Cis & Trans Isomers)



Quantitative Results

Data A

	Name	Ret. Time	Area	Height	Conc.	Area %
1	RT9.491 Trans	9.491	15037833	1220682	0.000	56.334
2	RT11.721 Cis	11.721	11249558	773890	0.000	42.142

Figure2- HPLC Chromatogram for the StandardResveratrol(Cis & Trans Isomers)

Figure (1) showed the HPLC chromatogram for the purified extracted resveratrol crystals, with two retention time of 9,494 minutes and 11.73 minutes, indicating the two

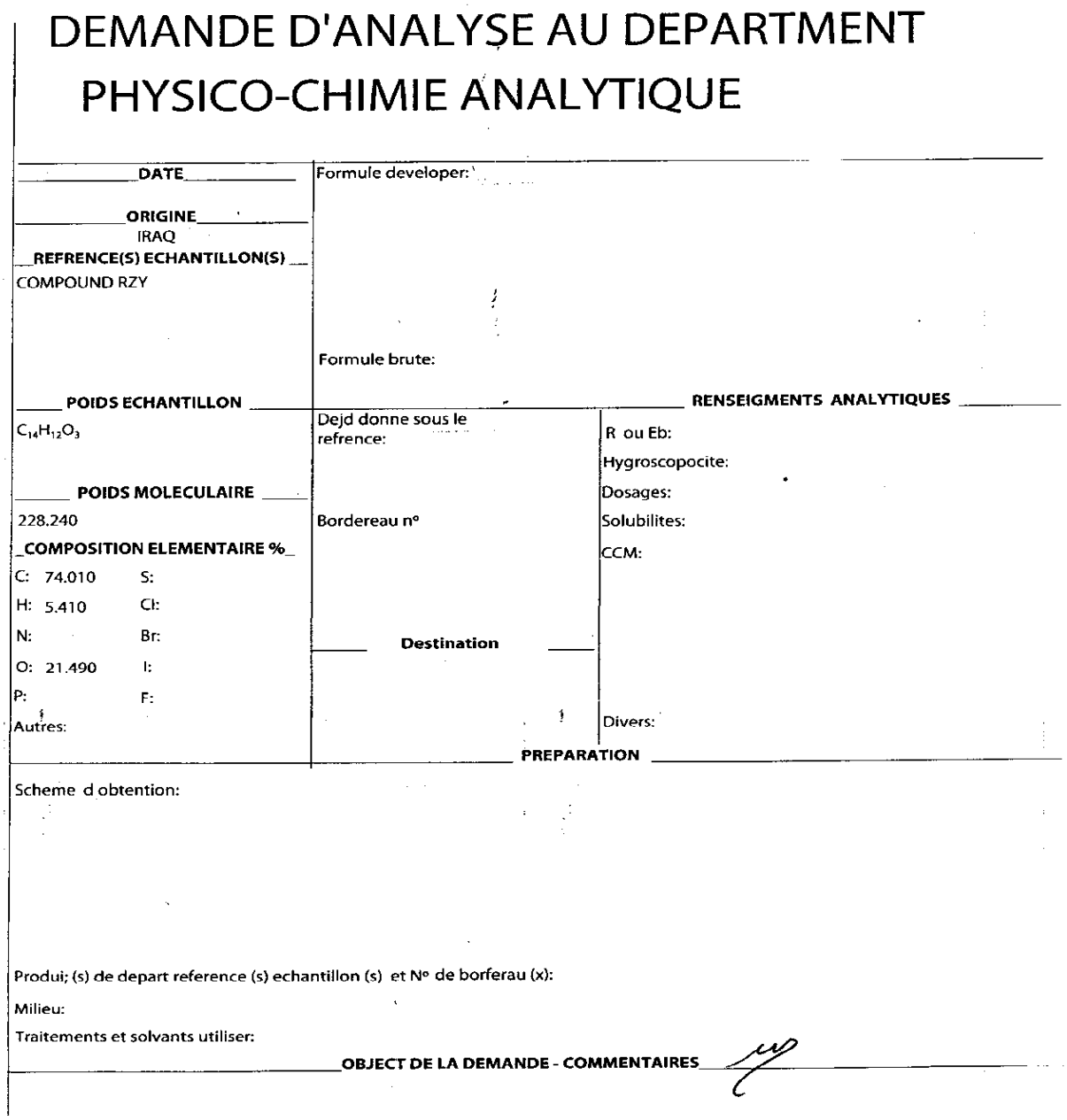
stereoisomers Trans and Cis, respectively. As for the figure (2), it indicates the retention time of the same two isomers Trans and Cis (9,491 and 11,721) respectively for Resveratrol standard.

Assay for the content of Carbone, Hydrogen, Oxygen and Nitrogen content(CHO&N)

Figure 3-includes the result of examining the content of carbon, hydrogen and oxygen CHO. proceeded by College of Pharmacy/University of Baghdad .The chemical crystal content is: carbon 74,010%, hydrogen 5,410%, and oxygen 21,490 % and that it is free of nitrogen, chlorine, sulfur and other elements shown in Figure (3) to have the chemical formula of the compound $C_{14}H_{12}O_3$.

FTIR ASSAY

Figure 4-and Figure 5- are the result of infrared rays FTIR for the purified Resveratrol to detect the functional groups using: The result showed the presence of the following groups detected in Figure (4) and in comparison with the results of the standard resveratrol figure-5. The reactive phenol group in the region 3221 for crystals and 3294 for the standard substance, while the cyclic CH group: is appeared in the region 2937 for the crystals and 2931.6 for the standard substance. The C=C reactive group appeared in the region 1584 for pure crystals and the region of 1689.23 for the standard substance. C=C group aliphatic, this effective group appeared in the area of 2862.51, while this group was found in the standard substance in the area of 2850.79.



DEMANDE D'ANALYSE AU DEPARTMENT
PHYSICO-CHIMIE ANALYTIQUE

DATE

ORIGINE
IRAQ

REFRENCE(S) ECHANTILLON(S)
COMPOUND RZY

POIDS ECHANTILLON

$C_{14}H_{12}O_3$

POIDS MOLECULAIRE
228.240

COMPOSITION ELEMENTAIRE %
C: 74.010 S:
H: 5.410 Cl:
N: Br:
O: 21.490 I:
P: F:
Autres:

Formule developer:

Formule brute:

Dejd donne sous le refrence:

Bordereau n°

Destination

RENSEIGMENTS ANALYTIQUES
R ou Eb:
Hygroscopocite:
Dosages:
Solubilites:
CCM:
Divers:

PREPARATION

Scheme d obtention:

Produi; (s) de depart reference (s) echantillon (s) et N° de borferau (x):

Milieu:

Traitements et solvants utiliser:

OBJECT DE LA DEMANDE - COMMENTAIRES

Figure 3-The CHO &N analysis report for the purified extracted Resveratrol

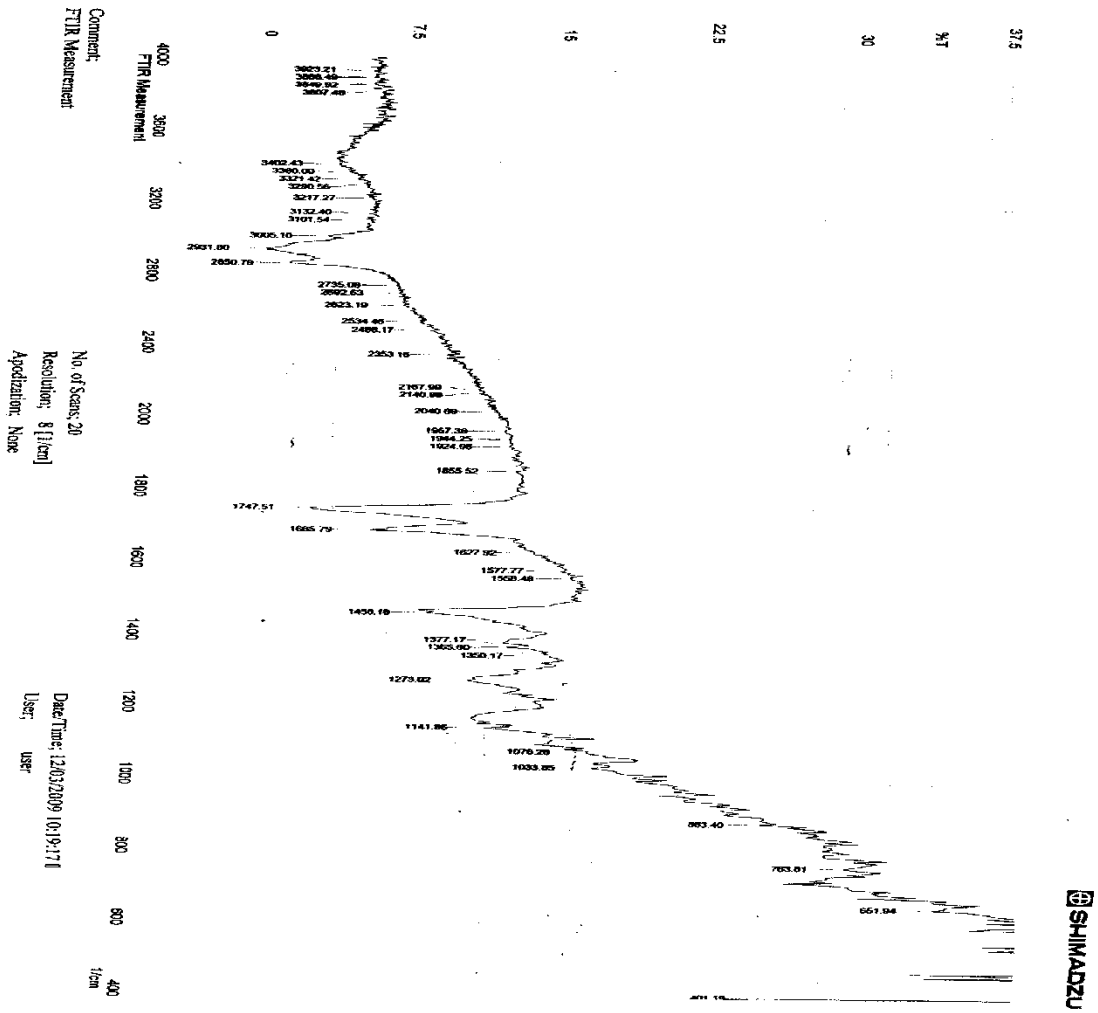


Figure-4-FTIR chart for the purified Resveratrol

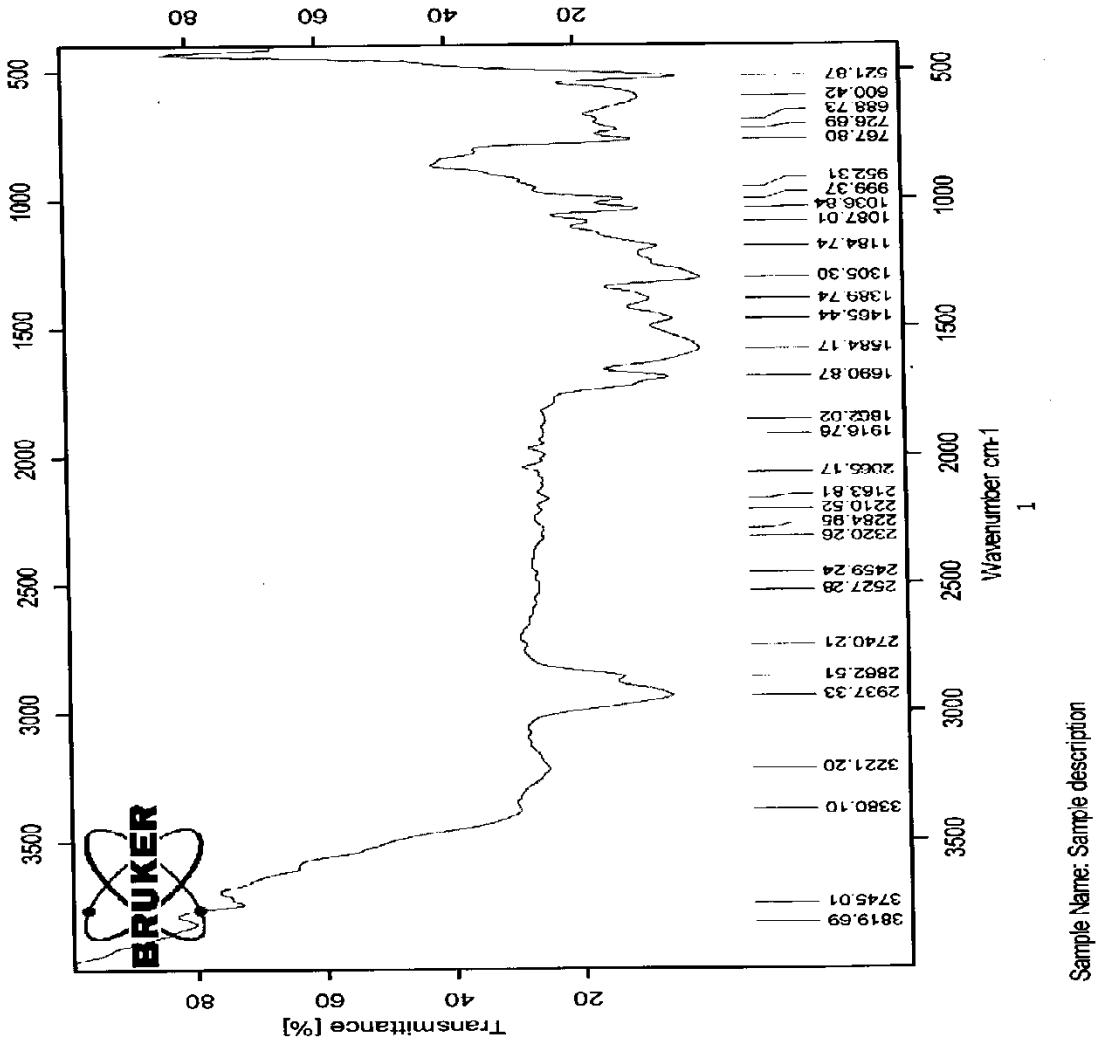


Figure-5-FTIR chart for Resveratrol Standard

2-Effect of Resveratrol on Normal Lymphocytes proliferations

Table-1 - illustrated the effect of different Resveratrol extracted from the local black grape on lymphocytes isolated from normal human blood cells. All concentrations affected the lymphocytes growth causing minor inhibition rate in different manner with different concentration. This made an impressionthat Resveratrol played important rule in directed immunity as an immune-modulatory agent.

Table-1-The effect of different concentration of Resveratrol extracted from the local black grape on normal human blood lymphocytes.

Resveratrol concentration (µg/ml)	Lymphocyte inhibition rate %
10	6 %
20	27.5 %

25	17.3 %
30	8 %
35	10 %
40	9 %
45	14 %
50	19 %
100	16 %
250	12 %
500	11 %
2500	26.5%

Effect of Resveratrol on interleukins level

The immune modulation effect of the purified resveratrol can be detected in Table 2- where the level of different interleukins secreted by the treated lymphocyte cells with different concentration from this substance during two times of exposurewas very clear.

Table-2-The Effect of Purified Resveratrol on some interleukins level secreted by normal human lymphocytes

Resveratrol mg/ml 2hours	**IL-2 Level pg/ml**	**IL-6 Level pg/ml**	**IL-10 Level pg/ml**	**IL-12 Level pg/ml**
0.1	5 d	77.5 a	0 b	365 b
0.03	95 c	70.85 b	0 b	640 a
0.006	160 b	66.19 bc	0 b	165 d
Control	390 a	64.35 c	41.3 a	480 b
LSD value	**32.95 ****	**6.702 ****	**5.240 ****	**71.63 ****
Resveratrol mg/ml 4hours				
0.1	105 b	86.30 a	6.167 c	175 c
0.03	105 b	78.42 b	60.50 b	400 a
0.006	85 b	71.13 c	67.50 a	310 b
Control	233.34 a	81.66 a	54.67 b	460 a

LSD value	47.82 **	6.319 **	6.011 **	62.956 **
Means having with the different letters in same column differed significantly. ** (P≤0.01).				

Resveratrol, is one of the chemical compounds that have the ability to regulate, modify and strengthen the immune functions of the immune system in the human body and has the ability to cause changes in the levels of cytokines that were selected for this experiment, namely (IL-2, IL-6, IL-10 and IL-12) compared with the levels of cytokines secreted by a control sample in cell culture. After two hours treating of cultured lymphocytes with resveratrol, it was observed that there is a significant increase in the production of both IL-6 (stimulates the growth and differentiation of T and B cells), and in IL-12 level significantly at 0.03ug/ml resveratrol (IL-12 has a role in activating the natural killing cells and differentiation of some NK cells) from CD + 4 T lymphocyte cells and transforming them into TH1-like cells as well as stimulating them to produce INF-gamma. IL-10 was not produced by the treated lymphocytes combined with significant decreasingof IL-2 compared with the control.While after four hours from lymphocytes exposure with the purified Resveratrol, there was a decrease in the levels of IL-2 without significantly. The expression of IL-10 was intiated after 4 hours from resveratrol exposure in comparision to normal level . The increase in production of the latter indicates that resveratrol has anti-inflammatory properties despite the low concentrations used in the experiment in relative to negative control[11].

Since IL-2 and IL-12 are products of the TH1 immune response, while IL-6, and IL-10 are secreted by lymphocytes through TH2 immune response, the results obtained from this experiment showed that the TH1 / TH2 ratio directed towards the TH1 cellular immune response for normal lymphocytes exposure to resveratrol[12-15] .

Accordingly, the resveratrol extracted from blackgrape skin has the ability to regulate and strengthen with boosting the immune system, to stand against diseases and fight abnormal cells in the body, as it works as an immunomodulation agent.

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